IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING NATIONAL PHASE OF
PCT APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

To: Hon. Commissioner of Patents
Washington, D.C. 20231

00909

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Atty Dkt: **PM** 276653 /PPD50360/UST
M# /Client Ref.

From: Pillsbury Winthrop LLP, IP Group:

Date: February 13, 2001

This is a **REQUEST** for **FILING** a PCT/USA National Phase Application based on:

1. International Application	2. International Filing Date	3. Earliest Priority Date Claimed
PCT/GB99/02652	12 AUG 1999	13 AUG 1998
⇧ country code	Day MONTH Year	Day MONTH Year
		(use item 2 if no earlier priority)

4. Measured from the earliest priority date in item 3, this PCT/USA National Phase Application Request is being filed within:

(a) ☐ 20 months from above item 3 date (b) ☒ 30 months from above item 3 date,

(c) Therefore, the due date (unextendable) is February 13, 2001

5. Title of Invention EXPRESSION OF BACTERIAL SIGNAL MOLECULES IN PLANTS

6. Inventor(s) FRAY, Rupert et al

Applicant herewith submits the following under 35 U.S.C. 371 to effect filing:

7. ☒ Please immediately start national examination procedures (35 U.S.C. 371 (f)).

8. ☒ **A copy of the International Application** as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (file if in English but, if in foreign language, file only if not transmitted to PTO by the International Bureau) including:

a. ☒ Request;
b. ☒ Abstract;
c. 13 pgs. Spec. and Claims;
d. 1 sheet(s) Drawing which are ☐ informal ☒ formal of size ☒ A4 ☐ 11"

9. ☒ **A copy of the International Application has been transmitted by the International Bureau.**

10. **A translation of the International Application** into English (35 U.S.C. 371(c)(2))
a. ☐ is transmitted herewith including: (1) ☐ Request; (2) ☐ Abstract;
(3) _____ pgs. Spec. and Claims;
(4) _____ sheet(s) Drawing which are:
☐ informal ☐ formal of size ☐ A4 ☐ 11"
b. ☐ is not required, as the application was filed in English.
c. ☐ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
d. ☐ Translation verification attached (not required now).

RE: USA National Filing of PCT/GB99/02652

24. Attached:

25. **Preliminary Amendment:**

25.5 Per Item 17.c2, **cancel original** pages #_____, claims #_____, Drawing Sheets #

26. **Calculation of the U.S. National Fee (35 U.S.C. 371 (c)(1)) and other fees is as follows:**
Based on amended claim(s) per above item(s) ☐ 12, ☐ 14, ☐ 17, ☐ 25, ☐ 25.5 (hilite)

Total Effective Claims	minus 20 =	x $18/$9	=	$0	966/967
Independent Claims	minus 3 =	x $80/$40	=	$0	964/965
If any proper (ignore improper) Multiple Dependent claim is present,		add$270/$135		+0	968/969

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): →→ **BASIC FEE REQUIRED, NOW** →→→→

A. If country code letters in item 1 are **not** "US","BR","BB","TT","MX","IL" "NZ", "IN" or "ZA"

See item 16 re:

1.	Search Report was not prepared by EPO or JPO - - - - - - - - -	add$1000/$500		960/961
2.	Search Report was prepared by EPO or JPO - - - - - - - - - - - -	add$860/$430	+860	970/971

SKIP B, C, D AND E UNLESS country code letters in item 1 are "US","BR","BB","TT","MX","IL", "NZ", "IN" or "ZA"

(X) →	☐	B.	If USPTO did not issue both International Search Report (ISR) and (if box 4(b) above is X'd) the International Examination Report (IPER), - - - - - - - - - - - - - - - - - - -	add$970/$485	+0	960/961
(only) (one) → (of)	☐	C.	If USPTO issued ISR but not IPER (or box 4(a) above is X'd), -	add$710/$355	+0	958/959
(these) (4) → (boxes)	☐	D.	If USPTO issued IPER but IPER Sec. V boxes not all 3 YES, -	add$690/$345	+0	956/957
→	☐	E.	If international preliminary examination fee was paid to USPTO and Rules 492(a)(4) and 496(b) satisfied (IPER Sec. V all 3 boxes YES for all claims), - - - - - - - - - - - - -	add $100/$50	+0	962/963

27. **SUBTOTAL =** $860

28. If Assignment box 19 above is X'd, add Assignment Recording fee of ----$40 +0 (581)

29. Attached is a check to cover the - **TOTAL FEES** $860

Our Deposit Account No. 03-3975
Our Order No. 70596 (C#) | 276653 (M#)

00909

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 and 492 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.
This CHARGE STATEMENT does not authorize charge of the issue fee until/unless an issue fee transmittal form is filed

Pillsbury Winthrop LLP
Intellectual Property Group

By Atty: Paul N. Kokulis **Reg. No.** 16773
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NOTE: File in duplicate with 2 postcard receipts (PAT-103) & attachments.

RE: USA National Filing of PCT /GB99/02652

11. ☒ **PLEASE AMEND** the specification before its first line by inserting as a separate paragraph:
a. ☒ --This application is the national phase of international application PCT/GB99/02652 filed August 12, 1999 which designated the U.S.--
b. ☐ --This application also claims the benefit of U.S. Provisional Application No. 60/ _____, filed _____ .--

12. ☐ Amendments to the claims of the International Application **under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., before 18th month from first priority date above in item 3, are transmitted herewith (file only if in English) including:**

13. ☒ PCT Article 19 claim amendments (if any) have been transmitted by the International Bureau

14. ☐ Translation of the amendments to the claims **under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., of claim amendments** made before 18th month, **is attached (required by 20th month from the date in item 3 if box 4(a) above is X'd, or 30th month if box 4(b) is X'd, or else amendments will be considered canceled).**

15. **A declaration of the inventor** (35 U.S.C. 371(c)(4))
a. ☐ is submitted herewith ☐ Original ☐ Facsimile/Copy
b. ☒ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.

16. **An International Search Report (ISR):**
a. Was prepared by ☒ European Patent Office ☐ Japanese Patent Office ☐ Other
b. ☒ has been transmitted by the international Bureau to PTO.
c. ☒ copy herewith (2 pg(s).) ☒ plus Annex of family members (1 pg(s).).

17. **International Preliminary Examination Report** (IPER):
a. ☒ has been transmitted (if this letter is filed after 28 months from date in item 3) in English by the International Bureau with Annexes (if any) in original language.
b. ☒ copy herewith in English.
c.1 ☐ IPER Annex(es) in original language ("Annexes" are amendments made to claims/spec/drawings during Examination) including attached amended:
c.2 ☐ Specification/claim pages #_____ claims #
Dwg Sheets #
d. ☐ Translation of Annex(es) to IPER **(required by 30th month due date, or else annexed amendments will be considered canceled).**

18. **Information Disclosure Statement** including:
a. ☐ Attached Form PTO-1449 listing documents
b. ☐ Attached copies of documents listed on Form PTO-1449
c. ☒ A concise explanation of relevance of ISR references is given in the ISR.

19. ☐ **Assignment** document and Cover Sheet for recording are attached. Please mail the recorded assignment document back to the person whose signature, name and address appear at the end of this letter.

20. ☐ Copy of Power to IA agent.

21. ☐ **Drawings** (complete only if 8d or 10a(4) not completed): ___ sheet(s) per set: ☐ 1 set informal; ☐ Formal of size ☐ A4 ☐ 11"

22. Small Entity Status ⌀ ☒ is **Not** claimed ☐ is claimed (**pre**-filing confirmation required)
22(a) _____ (No.) Small Entity Statement(s) enclosed (since 9/8/00 Small Entity Statements(s) not essential to make claim)

23. **Priority** is hereby claimed under 35 U.S.C. 119/365 based on the priority claim and the certified copy, both filed in the International Application during the international stage based on the filing in (country) Great Britain of:

	Application No.	Filing Date		Application No.	Filing Date
(1)	9817707.4	13 AUG 1998	(2)		
(3)			(4)		
(5)			(6)		

a. ☒ See Form PCT/IB/304 sent to US/DO with copy of priority documents. If copy has not been received, please proceed promptly to obtain same from the IB.
b. ☐ Copy of Form PCT/IB/304 attached.

EXPRESSION OF BACTERIAL SIGNAL MOLECULES IN PLANTS

This invention relates to the expression of bacterial signal molecules in plants which allows, for example, modulation of the interaction between plants and infecting or symbiotic bacteria.

The ability of bacteria to respond to environmental cues such as nutrient availability, temperature or pH is critical to microbe success. It is apparent that individual bacteria can also sense the density and state of the local bacterial population of which they are members. This sensing ability, referred to as "quorum sensing", allows a bacterial community to synchronise growth and development and, when the minimum population or "quorum" has been achieved, to initiate a concerted population response. Quorum sensing is thus an example of multicellular behaviour in prokaryotes and regulates diverse physiological processes including bioluminescence, swarming, antibiotic biosynthesis, plasmid conjugal transfer and the production of virulence determinants in pathogens.

The signalling pheromones upon which quorum sensing is based have been identified as *N*-acyl-L-homoserine lactones (reviewed by Swift *et.al.* "Quorum sensing: a population-density component in the determination of bacterial phenotype", *Trends in Biochemical Science*, **21**, 214-219 (1996). *N*-acyl-L-homoserine lactones molecules comprise a homoserine lactone moiety (derived from amino acid metabolism, possibly via S-adenosyl methionine) linked to an acyl sidechain (probably derived from fatty acid synthesis). A number of *N*-acyl-L-homoserine lactones with different acyl side chains have been identified in different bacterial systems where they elicit a wide range of quorum-dependent responses such as swarming, pathogenicity, conjugation or production of colour, light or antibiotics.

Several bacterial species produce the same *N*-acyl-L-homoserine lactone, although in some of the species it may regulate a different biological process. For example, the *luxI* gene product of *Photobacterium fischeri* synthesises *N*-(3-oxohexanoyl)-L-homoserine lactone which regulates bioluminescence in a cell density-dependent manner, whilst the *carI* gene product of *Erwinia carotovora* also produces *N*-(3-oxohexanoyl)-L-homoserine lactone which in this bacterium is responsible for the induction of secreted plant cell wall degrading exoenzymes and of the antibiotic carbapenem. The *cviI* gene of *Chromobacterium violaceum* encodes the enzyme for synthesis of *N*-hexanoyl-L-homoserine lactone which is

structurally very similar to the oxohexanoyl analogue and which induces production of the purple pigment violacein. Inactivation of *luxI, carI* or *cviI* results in loss of the density dependent bioluminescence, virulence or violacein production respectively. The relevant operons can, however, be induced by the addition of an exogenous supply of the *N*-acyl-L-homoserine lactone to the mutant bacteria.

CarI mutants of *Erwinia carotovora* appear to be completely avirulent when tested on tobacco. They can neither macerate plant tissue nor multiply *in planta* because they lack pectin lyase, pectate lyase, polygalacturonase, cellulase and protease. It is pertinent to ask how the expression of these exoenzymes only at high cell density in the wild-type cells may contribute to the success of *Erwinia* as a plant pathogen. It has been suggested that under aerobic conditions, a successful *E.carotovora* infection requires a relatively high inoculum (10^6 - 10^7 c.f.u.) and the progression of the disease is then a competition between bacterial multiplication and development of plant resistance. Thus, the production of macerating enzymes at low cell densities would not give rise to a successful infection, but would result in the induction of the local and systemic plant defence response, which in turn would hamper subsequent infections. Such resistance to *E.carotovora* infection is seen when the plant defence response is artificially induced by the application of salicylic acid.

While not wishing to be bound by any theory as to the manner in which the invention proposed herein operates, the following explanation of the naturally occurring phenomenon of quorum sensing is offered. Using *Photobacterium fischeri* as a convenient example, the expression of two regulatory genes, *luxI* and *luxR*, is necessary for the expression of the genes necessary for bioluminescence. Expression of *luxI* leads to production of the pheromone *N*-(3-hydroxyl)hexanoyl-L-homoserine lactone, the mechanisms by which the lactone is synthesised being largely irrelevant to this discussion. A complex of the pheromone with the protein produced by the *luxR* gene gives a phenotypic response, in the case of *P.fischeri*, bioluminescence. At low population density of bacteria, *luxI* and *luxR* are transcribed at low level and there is insufficient accumulation of the pheromone (*N*-acyl-L-homoserine lactone) to elicit *luxI*-dependent transcription of the operon responsible for visible bioluminescence. It has been suggested that in the absence of sufficient pheromone, and/or a chaperonin known as GroESL, *luxR* is unstable and sensitive to degradation. As the population grows, however, the concentration of the pheromone increases gradually. At a critical level of the pheromone, which represents a critical population density, a complex between *luxR* and the pheromone is thought to bind to a palindromic sequence within the *luxI*

operator thereby activating increased transcription of the operon necessary for increased production of the pheromone and for bioluminescence.

The present invention seeks to provide a method and means of manipulating plant/microbe interactions.

According to the present invention there is provided a method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria, comprising introducing into the genome of the plant by transformation the ability to synthesise a *N*-acyl-L-homoserine lactone.

Further according to the invention there is provided a method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria, comprising introducing into the genome of the plant by transformation the ability to synthesise an analogue of *N*-acyl-L-homoserine lactone.

The invention also provides a method of enhancing interaction between an antifungal rhizobacterium and a plant comprising introducing into the genome of the plant by transformation the ability to synthesise the *N*-acyl-L-homoserine lactone naturally produced by the rhizobacterium.

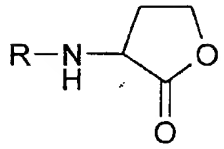
The invention also provides a recombinant plant genome containing a gene construct for *in planta* expression of an *N*-acyl-L-homoserine lactone.

Preferably expression of introduced genes is targeted to plant chloroplasts.

The gene specifying the N-acyl-L-homoserine lactone may be selected from the group consisting of, the *yenI* gene of *Yersinia enterocolitica;* the *cviI* gene of *Chromobacterium violaceum;* the *luxI* gene of *Photobacterium fischeri;* the *carI* gene of *Erwinia carotovora;* the *traI* gene of *Agrobacterium tumefaciens* and the *lasI* and *vsmI* genes of *Pseudomonas aeruginosa.*

Examples of suitable sources of DNAs specifying *N*-acyl-L-homoserine lactones and the acyl group involved are as follows:

Table 1



***N*-acyl-L-homoserine lactone**

Bacterium	Signal Generator	Response Regulator	*N*-acyl-group R
Yersinia enterocolitica	*yenI*	*yenR*	3-oxohexanoyl
Chromobacterium violaceum	*cviI*	*cviR*	3-hexanoyl
Photobacterium fischeri	*luxI*	*luxR*	3-oxohexanoyl
Erwinia carotovora	*carI*	*carR*	3-oxohexanoyl
Agrobacterium tumefaciens	*traI*	*traR*	3-oxo-octanoyl
Pseudomonas aeruginosa	*lasI*	*lasR*	3-oxo-dodecanoyl
Pseudomonas aeruginosa	*vsmI*	*vsmR*	butanoyl

These examples in Table 1 are quoted in Swift *et.al.*, *Trends in Biochemical Science*, **21**, 214-219 (1996).

Table 2 below gives further examples along with references and the appropriate GenBank Accession Numbers.

Table 2

Organism	Signal generator	Response Regulator	Signal Molecule	GenBank Accession number	References
Aeromonas hydrophila	*AhyL*	*AhyR*	unknown	X89469	
Agrobacterium tumefaciens	*TraI*	*TraR*	*N*-(3-oxo)-octanoyl-L-homoserine Lactone (OOHL)	L17024, L22207	Fuqua et.al, 1994; Hwang et.al. 1995
Chromobacterium violaceum	*CviI*	*CviR*	*N*-hexanoyl-L-homoserine lactone (OHL)		Winson,et.al.., (1994)
Enterobacter	*EagI*	unknown	*N*-(-3-	x74300	Swift *et al.*, 1993

agglomerans			oxo)hexanoyl-L-homoserine lactone (OHHL)		
Erwinia carotovora subsp carotova	*CarI*	*CarR*	OHHL	U17224, X72891, X74299, X80475	McGowan et.al., 1995
Erwinia stewartii	*EsaI*	*EsaR*	OHHL	L32183, L32184	Beck von Bodman and Farrand, 1995
Escherichia coli	unknown	*SdiA*	unknown	Xo3691	Sitnikov *et al* 1995
Photobacterium fischeri	*LuxI*	*LuxR*	OHHL,OOHL	M19039, M96844, M25752	Meignhen, 1994; Devine *et al*, 1988
Pseudomonas aeruginosa	*LasI*	*LasR*	*N*-(-3-oxo)-dodecanoyl-L-homoserine lactone (OdDHL)	M59425	Winson *et al* 1995;.
	VsmI	*vsmR*	*N*-butanoyl-L-homoserine lactone (BHL), HHL	L08962, U11811, U15644	Winson *et al.*, 1995; Latifi *et al* 1995; Ochsner and Reiser, 1995.
Pseudomonas aureofaciens	*PhzI*	*PhzR*	unknown	L32729, L33724	Wood and Piersen, 1996
Rhizobium leguminosarum	unknown	*RhiR*	*N*(-3-hydroxy)-tetradecanoul-L-homoserine lactone (HtDeHL)	M98835	Fuqua *et al.*, 1994; Gray *et al.*, 1996.
Serratia liquefaciens	*SwrI*	unknown	BHL	U2823	
Aeromonas hydrophila	*ahyI*	*ahyR*	BHL		Swift et al., 1997
Aeromonas salmonicida	*AsaI*	unknown	BHL, *N*-hexanoyl-L-homoserine lactone		Swift *et al.*, 1997
Vibiro	*vanI*	*vanR*	*N*-(3-oxo-		Milton *et al.*,

anguillarum			decanoyl)-L-homoserine lactone (ODHL)		1997
Vibrio harveyi	*LuxLM*	*LuxN*	*N*-(3-hydroxy)-butanoyl-L-homoserine lactone (HBHL)	L13940	Meighen, 1994; Bassler *et al.*, 1994.
Yersinia enterocolitica	*YenI*	*YenR*	OHHL,HHL	X76082	Throup *et al.*, 1996.

References: Bassler et.al. *Molecular Microbiology*, **12**, 403-412 (1994)
Beck et.al. *J.Bacteriol.*, **177**, 5000-5008 (1995)
Devine et.al. *Biochemistry*, **27**,837-842 (1988)
Fuqua et.al. *J.Bacteriol.* **176**, 269-275 (1994)
Gray et.al. *J.Bacteriol.* **178**, 372-376 (1996)
Hwang et.al. *J.Biotech.* **177**, 449-458 (1995)
Latifi et.al. *Molecular Microbiology*, **17**, 333-343(1995)
McGowan et.al.*Microbiology*, **141**, 541-550 (1995)
Meignhen *Ann. Rev. Genet.* **28**,117-139(1994)
Milton et.al. *J.Bacteriol.* **179**, 3004-3012 (1994)
Ochsner and Reiser *Proc.Natl.Acad.Sci.USA*, **92**, 6424-6428 (1995)
Sitnikov et.al. *Molecular Microbiology*, **17**, 801-812 (1995
Swift et.al. *Molecular Microbiology*, **10**, 511-520 (1993)
Swift et.al. *J.Bacteriol.* **179**, 5271-5281 (1997)
Throup et.al. *Molecular Microbiology*, **17**, 345-356 (1996)
Winson et.al. *Proc.Natl.Acad.Sci USA*, **92**, 9427-9431 (1995)
Wood and Piersen, *Gene* 168, **49**-53 (1996)

Our invention is founded on our reasoning that if the inoculating bacteria were to encounter levels of *N*-acyl-L-homoserine lactone that gave a false indication of the local population size, the course of the ensuing infection would be drastically reduced.

A second aspect of the invention concerns engineering the plant to take advantage of the potential protective effect of antifungal rhizobacteria. There exist in the rhizosphere certain bacteria which are capable of attacking potential pathogenic fungal microorganisms which are also present in the soil, perhaps the best known of which are certain strains of *Pseudomonas fluorescens* and *P. aureofaciens*. But the population of such antifungal bacterial strains in the soil will normally be low and their antifungal activity dependent on the quorum sensing phenomenon to be activated. By imparting to the plant the ability to produce the activator molecule, the *N*-acyl-L-homoserine lactone, appropriate to the antifungal bacteria the antifungal activity may be initiated at low colony size providing earlier than normal protection of the plant against the pathogenic fungi. The rhizosphere-

expressed genes of the *rhiABC* operon of the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*, for example, are regulated by an AHL with a C14 side chain containing hydroxylated carbon in the 3 position and a single carbon-carbon double bond.

Transgenic plants producing an AHL signal molecule enhance the establishment of an antifungal environment on the rhizosphere. This phenomenon would also enable the use of disarmed bacterial strains to be used as crop protection biocontrol agents in conjunction with the AHL-producing transgenic plants.

The invention will now be described in the following Examples

The ability of AHLs to induce changes in neighbouring bacteria was tested in four ways;

(1) the ability of the AHLs to diffuse out of intact leaves was demonstrated by placing intact transgenic leaves on agar and subsequently removing it before overlaying with *C.violaceum* CV026 (see Example 4 below) and the outline of the whole leaf could be seen showing that the AHL diffused out of the leaf surface and not just the cut stem;

(2) being interested in whether the AHLs were only produced in the chloroplasts or whether they could be found in other tissues such as roots, the ability of the AHLs to diffuse from the roots was demonstrated in that AHLs in the vicinity of the roots were able to induce bioluminescence in a recombinant *E,coli* strain carrying an AHL-inducible operon: this showed that the root plastids are competent to suynthesise the AHLs are, alternatively, that the AHLs synthesised in green tissue can be transported to the roots but in either case the roots were clearly capable of signalling to nearby bacteria.

(3) AHL-producing plant tissue is capable of restoring G.graminis growth-inhibiting activity to the disarmed *P.aureofaciens* 30-84 *phzI-* strain (see Example 9 below)

(4) *Erwinia carotovora carI* (*expI*) mutants, which have greatly reduced virulence in their natural host plants were shown to infect transgenic tobacco plants which are producing AHLs (see Example 10 below).

Figure 1 herewith shows the components of the constructs pBDHEYI and pBDHERBYI described in the Examples.

Example 1

Preparation of pBDHEYI

pBDHEYI was constructed by fusing the alfalfa mosaic virus (AMV) translation enhancer sequence from pBI526 (Datla et.al., *Plant Science* **94**, 139-149 (1993)) to the *yenI*

coding sequence from *Yersinia enterocolitica*. The *yenI* sequence had previously been amplified by PCR to create an *Nco*I site overlapping the translation initiation sequence. This changed the second amino acid from leucine to valine but did not affect the ability of the encoded enzyme to synthesise *N*-acyl-L-homoserine lactones in a bacterial assay. The AMV/*yenI* fusion was cloned on a *Bgl*II/*Bam*HI fragment into the *Bam*HI of pDH51 (Pietrzak *et.al.*, *Nucl. Acids Res.* **14**, 5857-5868(1986)) to give pDHEYI. An *Eco*RI fragment of pDHEYI was cloned into the *Eco*RI site of pBIN19 (Bevan, *Nucl. Acids Res.* **12**, 8711-8721 (1984)) to give pBDHEYI.

Example 2

Preparation of pBDHERBYI

pBDHERBYI was constructed by fusing the petunia SSU611 ribulose bisphosphate carboxylase small subunit (*rbcS*) chloroplast targeting sequence (Dean et.al. *Mol, Gen. Genet.*, 206, 465-474 (1987)) to the AMV translation enhancer sequence of pBI526. An *Nco*I site was engineered to overlap the initiating ATG codon of *rbcS*. An *Sph*I site was engineered to overlap the initiating ATG codon of *yenI* and the *yenI* coding sequence cloned into the *Sph*I site of the SSU611 fragment. This site spans the cleavage site of the encoded chloroplast transit peptide. The AMV/*rbcS/yenI* fusion was cloned on a *Bgl*II/*Bam*HI fragment into the *Bam*HI site of pDH51 to give pDHERBYI. An *Eco*RI fragment from pDHERBYI was cloned into the *Eco*RI site of pBIN19 to give pBDHERBYI.

The rationale for producing pBDHERBYI and believing that it would be active in chloroplasts was as follows: in *E.coli* homoserine lactone is not produced by mutants of the threonine biosynthetic pathway that are blocked prior to homoserine synthesis but is produced by those mutants when supplied with an exogenous source of homoserine. However, *TraI*, the *N*-acyl-L-homoserine lactone biosynthetic enzyme in *Agrobacterium tumefaciens*, has been found to utilise *S*-adenosylmethionine and not homoserine as a substrate *in vitro*. There is also evidence for the acyl moiety being derived from fatty acid biosynthetic intermediates. In plants the enzymes of the threonine biosynthetic pathway are located in the chloroplast and this organelle is also active in fatty acid metabolism. Therefore the chloroplasts may be expected to contain the necessary precursors for *N*-acyl-L-homoserine lactone synthesis by *yenI* and more closely approximate to the environment in which *yenI* is normally active than would be the cytoplasm.

Example 3

Generation of Transgenic Plants

Construct pBDHEYI.for Example 1 and pBDHERBYI from Example 2 were transferred to the *Agrobacterium tumefaciens* strain LBA 4404 and used to transform tobacco leaf discs according to standard protocol (Draper et.al., pages 69-160, In Plant Genetic Transformation and gene expression: a laboratory manual; Draper et.al. (Eds) Blackwell Scientific Publications, London (1988)) .

The transgenic status of the resulting kanamycin positive explants was confirmed by Southern analysis (data not given)

Example 4

Complementation of Violacein Production

Leaf segments of the transgenic plants produced in Example 3 were tested for their ability to synthesise *N*(3-oxohexanoyl)-L-homoserine lactone or a related analogue.

A transgenic tobacco leaf was placed in an agar plate overnight. The leaf was then removed and the *cviI* mutant of *Chromobacterium violaceum* spread over the plate. Violacein production by the bacteria could be seen where the *N*-(3-oxohexanoyl)-L-homoserine lactone had diffused out of the leaf and into the agar.

Two leaf segments tested positive as indicated by the ability of a diffusible product to complement *C.violaceum*, inducing the production of the purple pigment violacein by the bacteria.

Example 5

Complementation of *carI*

Construct pBDHERBYI (Example 2) was transferred to the *Agrobacterium tumefaciens* strain LBA 4404 and transformed into tobacco. Leaf segments were tested for their ability to synthesise *N*(3-oxohexanoyl)-L-homoserine lactone or a related analogue.

An untransformed control and a transgenic BDHERBYI tobacco leaf were inoculated with *Erwinia carotovora* mutant for *carI*. The bacteria were applied at a high culture density (OD600 of 2.5) in a volume of 10 µl to a small wound site made with a hypodermic needle. A second BDHERBYI leaf was mock inoculated with bacterial culture medium alone.

The leaves were inspected after four days. The untransformed control and the mock inoculated leaf remained substantially unchanged. The sample inoculated with *E.carotovora* displayed advanced disease symptoms demonstrating that the pathogen can perceive and respond to the *N*-acyl-L-homoserine lactone being made by the transgenic plant.

Example 6

Complementation of *luxI*

Following a similar protocol as described above, the *luxR* *N*-acyl-L-homoserine lactone response regulator and the *lux* operon (minus *luxI)* of *Pseudomonas fischeri* was inserted into *E.coli*. When transgenic tobacco carrying the BDHERBYI construct was challenged with the *E.coli*, bioluminescence was induced in the bacteria demonstrating that the *luxR* gene was able to respond to the *N*-acyl-L-homoserine lactone produced by the plant.

Twenty-nine tobacco plants that were independently transformed with either BDHERBYI or BDHEYI were challenged with *C.violaceum* mutant for *cviI* (Example 3) and *E.coli* carrying an *N*-acyl-L-homoserine lactone-inducible *lux* operon. Table I summarises the results.

TABLE 1

	Number of plants				
	Positive reaction		**Negative reaction**		
Construct	***cviI***	***luxI***	***cviI***	***luxI***	**Total**
BDHERBYI	8	8	5	5	13
BDHEYI	0	0	16	16	16

Example 7

Extraction and TLC analysis of AHLs

For thin-layer chromatographic analysis, transgenic plant extracts were made by grinding two grams of plant tissue to a fine powder in liquid nitrogen and mixing the frozen powder with 200ml of warn distilled water. After five minutes, solid matter was filtered off and the filtrate extracted with an equal volume of ethyl acetate. The ethyl acetate layer was then dried over anhydrous magnesium sulphate, filtered and evaporated to dryness. The residue was taken up in 500 µl of acetonitrile and 20µl of this was applied to a C18 reverse phase TLC plate (Merck). A similar extract from an untranformed control plant was also spotted on to the plate. *N*-hexanoyl-L-homoserine lactone (HHL) ($1x10^{-8}$g) and *N*-(3-oxohexanoyl) -L homoserine lactone (OHHL) ($5x10^{-7}$g) were applied as standards and the chromatogram developed with methanol/water (60:40 vol/vol) as running solvent (Shaw P.D. *et.al.* Proc.Natl.Acad,Sci. USA 94: 6036-6041 (1997)). After drying, AHLs were located on the TLC plate by overlaying *C.violaceum* strain CV026 in top agar as described by McClean *et.al.* (Microbiology-UK, 143: 3703-3711 (1997). After 16 hours growth at 28°C the presence of AHLs was indicated by localised violacein production.

Two different molecules with R_f values identical to the synthetic HHL and OHHL standards were observed.

Example 8

HPLC and LC-MS Analyses

For HPLC and LC-MS analyses, transgenic plant extracts were made by grinding the issue in ethyl acetate. The supernatant was taken and the plant residue re-extracted with ethyl acetate, the supernatants pooled and the process repeated until the plant residue was white/brown n colour and free of chlorophyll. The ethyl acetate layer was separated from a small plant-derived aqueous layer and dried over anhydrous magnesium sulphate, filtered, and evaporated to dryness. The residue was resuspended in 500 µl of methanol, this was brought to 60% methanol with sterile distilled water and placed at -20°C overnight to precipitate out the majority of the chlorophyll. After pelleting any solid matter by centrifugation in a bench-top microfuge, the AHL-containing supernatant was partitioned against 10 volumes of ethyla acetate and the organic phase evaporated to dryness. The residue was taken up into 500µl of acetonitrile. For both LC-MS and HPLC analyses linear gradients of acetonitrile in water were run (20-100% over 32 minutes) as described by Camara *et.al.* In Methods in Microbiology: Bacterial Pathogenesis Vol. 27: 319-330, Williams *et.al.* (Eds) (1998). OHHL and HHL eluted at 9 minutes and 13.5 minutes respectively.

The presence of HHL and OHHL, detected in TLC analysis, were confirmed.

Example 9

Assay for restoration of activity to *P.aureofaciens* mutant

Leaf material from transgenic and non-transformed control plants were placed in wells cut in a potato dextrose agar plate (Oxoid). *P.aureofaciens* strain 80-84I (*phzI*$^-$) was inoculated adjacent to the wells and the plates incubated for 24 hours at 22°C. The *G.graminis* var. tritici was then introduced on the opposite side of the plate and the whole incubated for a further four days.

The antifungal activity of the *P.aureofaciens phzI*$^-$ strain against the *G.graminis* was found to have been restored.

Example 10

Assay for restoration of virulence to *Erwinia carotovora* avirulent mutant

Untransformed and control BDHERBYI tobacco leaves were inoculated with the avirulent E.carotovora mutant PNP22 (Bainton *et.al.*, Biochem.Journal, **288**: 997-1004

(1992) and also Jones *et.al.*, The EMBO Journal, **12**: 2477-2482 (1993)) The bacteria were applied at high culture density (OD600=2.5) in a volume of 10µl to a small wound site made with a hypodermic needle.

Normally these *Erwinia* mutants are avirulent in the tobacco system, in which they can neither macerate plant tissue nor multiply *in planta* because they are defective in the production of plant cell-wall-degrading enzymes pectin lyase, pectate lyase, polygalacturonase, cellulase and protease. The regulated expression of plant cell wall-degrading enzymes only at high density in wild-type bacteria may contribute to the success of *Erwinia* as a plant pathogen. Under aerobic conditions, *E.carotovora* infection only occurs when the bacteria has reached sufficiently high population densitiy such that disease progression depends on competition between bacterial multiplication and the plant host defences. Thus the production of macerating enzymes at low cell densities would not give rise to a successful infection, but would result in the premature induction of the local and systemic plant defence response, which in turn would hamper subsequent infection. Thus, if the infecting pathogen were to encounter AHL levels that gave a false indication of the local bacterial population size the course of the ensuing infection will be substantially reduced as the plant is able to mount a successful defence to a weak attack.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

CLAIMS

1. A method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria, comprising introducing into the genome of the plant by transformation the ability to synthesise a *N*-acyl-L-homoserine lactone.

2. A method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria, comprising introducing into the genome of the plant by transformation the ability to synthesise an analogue of *N*-acyl-L-homoserine lactone capable of competing with the *N*-acyl-L-homoserine lactone secreted by infecting bacteria for *N*-acyl-L-homoserine lactone receptor sites therein.

3. A method of enhancing interaction between a rhizobacterium and a plant comprising introducing into the genome of the plant by transformation the ability to synthesise the *N*-acyl-L-homoserine lactone naturally produced by the rhizobacterium.

4. A method as claimed in any of claims 1 to 3 in which the gene expressing the N-acyl-L-homoserine lactone is selected from the group consisting of, the *yenI* gene of *Yersinia enterocolitica;* the *cviI* gene of *Chromobacterium violaceum;* the *luxI* gene of *Photobacterium fischeri;* the *carI* gene of *Erwinia carotovora;* the *traI* gene of *Agrobacterium tumefaciens* and the *lasI* and *vsmI* genes of *Pseudomonas aeruginosa.*

5. A recombinant plant genome containing a gene construct for *in planta* expression of an *N*-acyl-L-homoserine lactone and/or the response regulator thereof.

6. A genome as claimed in claim 5 in which expression of the said *N*-acyl-L-homoserine lactone is targeted to plant chloroplasts.

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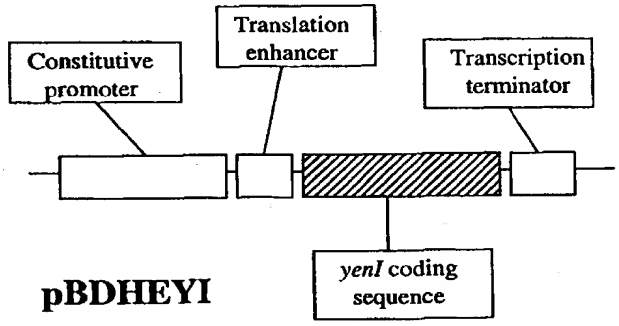
(54) **Title:** EXPRESSION OF BACTERIAL SIGNAL MOLECULES IN PLANTS

(57) **Abstract**

The ability of a plant to defend against attack by bacteria, and any virus borne by the bacteria, is enhanced by transforming the plant genome with a gene of bacterial origin which enables the plant to produce a bacterial pheromone, N–acyl–L–homoserine lactone. Such plants also secrete the lactone into the soil enhancing the protective effect of antifungal rhozobacteria.



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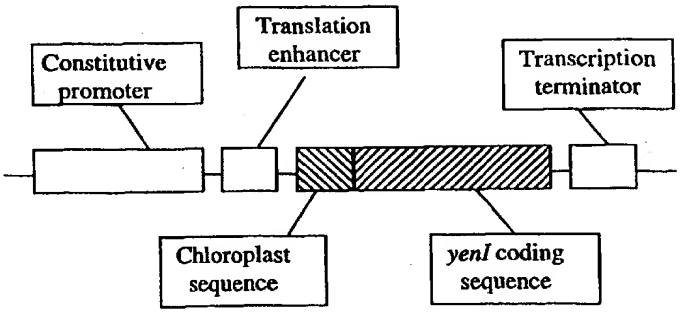

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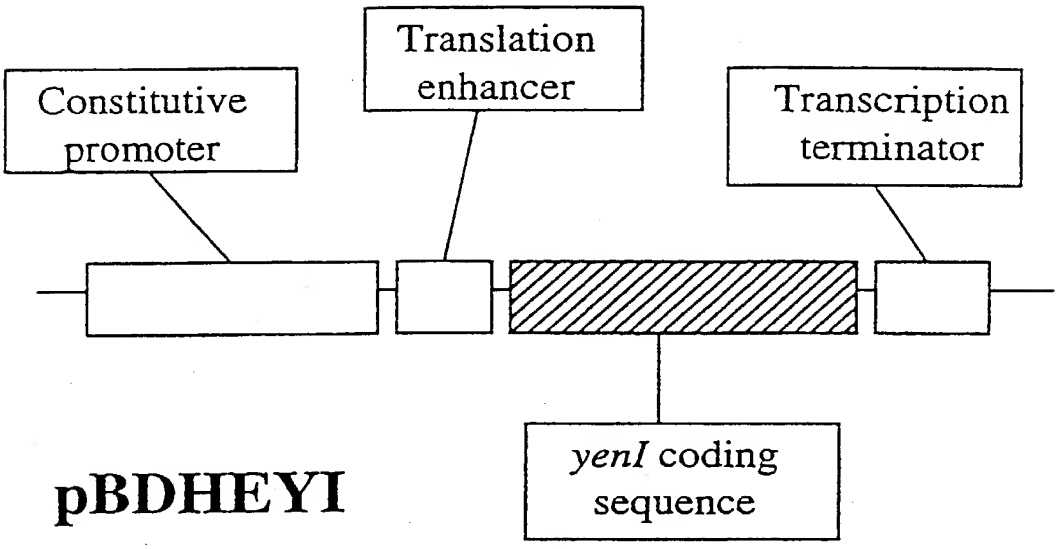
Figure 1



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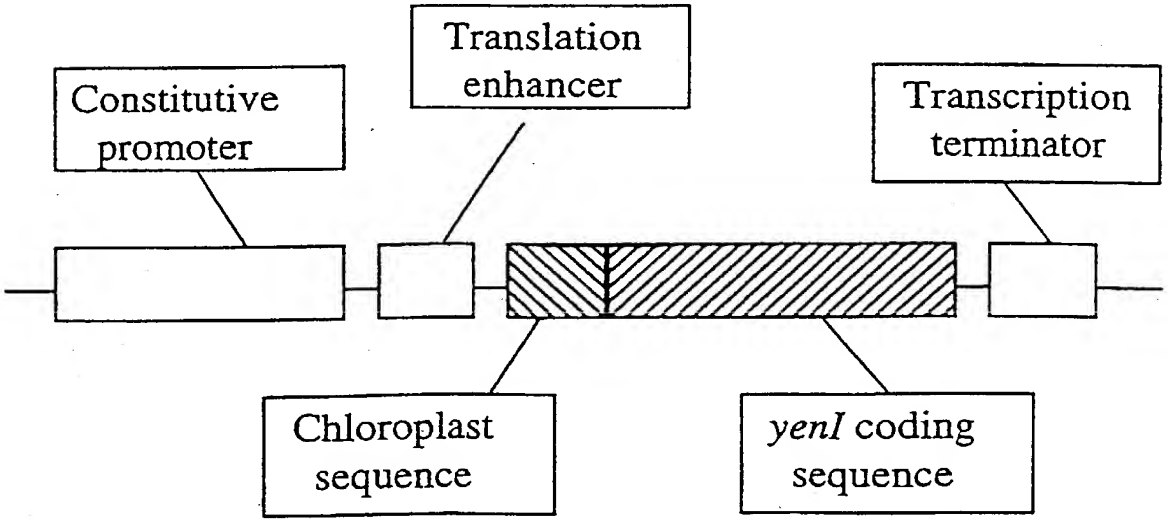

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the specification of which (CHECK applicable BOX(ES))

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→ B. ☐ was filed on _____ as U.S. Application No. _____ / _____

→ C. ☒ was filed as PCT International Application No. PCT/ GB99/02652 on August 12, 1999

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I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. Except as noted below, I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International Application which designated at least one other country than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International Application, filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

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9817707.4	Great Britain	13 AUG 1998			

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And I hereby appoint Pillsbury Winthrop LLP, Intellectual Property Group, telephone number (202) 861-3000 (to whom all communications are to be directed), and persons of that firm who are associated with USPTO Customer No. 909 (see below label) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete from that Customer No. names of persons no longer with their firm, to add new persons of their Firm to that Customer No., and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above Firm and/or an attorney of that Firm in writing to the contrary.

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☒ FOR ADDITIONAL INVENTORS see attached page.

☐ See additional foreign priorities on attached page (incorporated herein by reference).

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the specification of which (CHECK applicable BOX(ES))

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(include Zip Code)	19426		

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(5) **INVENTOR'S SIGNATURE:** Gordon Stewart **Date:** 10 May 2001

	Gordon	S.A.B.	**STEWART (deceased)**
	First	Middle Initial	Family Name
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	City	State/Foreign Country	Country of Citizenship
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(include Zip Code)			

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Residence			
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	First	Middle Initial	Family Name
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Mailing Address			
(include Zip Code)			

FOR UTILITY/DESIGN CIP/PLANT ORIGINAL/SUBSTITUTE DECLARATIONS

RULE 63 (37 C.F.R. 1.63) DECLARATION AND POWER OF ATTORNEY ⇨⇨⇨ BY LEGAL REPRESENTATIVE ⇦⇦⇦ FOR PATENT APPLICATION IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PW FORM

As a below named legal representative, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe the below listed to be the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

EXPRESSION OF BACTERIAL SIGNAL MOLECULES IN PLANTS

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the specification of which (CHECK applicable BOX(ES))

⇨ ☒ is attached hereto.

⇨ ☐ was filed on _____ as U.S. Application No. 0_/_____

⇨ ☒ was filed as PCT international Application No. PCT/ GB99/02652 on August 12, 1999

⇨⇨ and (if applicable to U.S. or PCT application) was amended on _____

Inventor(s) (NOTE: For deceased inventor(s), state last residence and country of citizenship)

1. Name: Rupert (First) George (Middle Initial) FRAY (Family Name) Great Britain (Country of Citizenship)
   Residence (City) Loughborough (State/Foreign Country) Great Britain

2. Name: John (First) Peter (Middle Initial) THROUP (Family Name) Great Britain (Country of Citizenship)
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3. Name: Andrew (First) David (Middle Initial) WALLACE (Family Name) Great Britain (Country of Citizenship)
   Residence (City) Bedfordshire (State/Foreign Country) Great Britain

4. Name: Donald (First) (Middle Initial) GRIERSON (Family Name) Great Britain (Country of Citizenship)
   Residence (City) Loughborough (State/Foreign Country) Great Briain

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5 Name: Gordon (First) S.A.B. (Middle Initial) STEWART (deceased) (Family Name) Great Britain (Country of Citizenship)
   Residence (City) Nottingham (State/Foreign Country) Great Briain

(FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet (PAT-116-2) for same information for each re name, citizenship, and residence.)

Of the foregoing inventor(s) Gordon S.A.B. STEWART is deceased but was late a citizen and resident as indicated and I am

X one) ⇨⇨ ☒ administrator/administratrix of his/~~her~~ estate, ☐ his/her heir, ☐ executor/executrix of his/her last will and testament

BOX) ⇨⇨ ☐ his/her guardian ☐ his/her conservator/conservatrix ☐ other

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. 1.56(a). I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate filed by me, the listed inventive entity, or assignee thereof disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

PRIOR FOREIGN APPLICATION(S)			Priority Claimed	
Number	Country	Day/MONTH/Year Filed	Yes	No
9817707.4	Great Britain	13 AUG 1998	X	

I hereby claim the benefit under 35 U.S.C. 119/120/365 of all United States applications listed below and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

PRIOR U.S. OR PCT APPLICATION(S) Application No. (series code/serial no.)	Day/MONTH/Year Filed	Status pending, abandoned, patented
PCT/GB99/02652	12 AUG 1999	pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Pillsbury Madison & Sutro LLP, Intellectual Property Group, telephone number (202) 861-3000 (to whom all communications are to be directed), and persons of that firm are associated with USPTO Customer No. 909 (see below label) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete from that customer No. names of persons no longer with their firm, to add new persons of their Firm to that Customer No., and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above Firm and/or an attorney of that Firm in writing to the contrary.

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1. LEGAL REPRESENTATIVE'S SIGNATURE: lesley A Stewart Date 10th May 2001

511 Name (typed) LESLEY (First Name) A (Middle Initial) STEWART (Family) UNITED KINGDOM (GREAT BRITAIN) (Country of Citizenship)

Residence (City) LOUGHBOROUGH (State/Foreign Country) UNITED KINGDOM (GREAT BRITAIN)

Post Office Address (Include Zip Code) GBX

2. LEGAL REPRESENTATIVE'S SIGNATURE: _____ Date _____

Name (typed) _____ (First Name) (Middle Initial) (Family) (Country of Citizenship)

Residence (City) _____ (State/Foreign Country) _____

Post Office Address (Include Zip Code) _____

3. LEGAL REPRESENTATIVE'S SIGNATURE: _____ Date _____

Name (typed) _____ (First Name) (Middle Initial) (Family) (Country of Citizenship)

Residence (City) _____ (State/Foreign Country) _____

Post Office Address (Include Zip Code) _____

4. LEGAL REPRESENTATIVE'S SIGNATURE: _____ Date _____

Name (typed) _____ (First Name) (Middle Initial) (Family) (Country of Citizenship)

Residence (City) _____ (State/Foreign Country) _____

Post Office Address (Include Zip Code) _____